Plant Cell Reports (1998) 17: 881–885

P. R. Salon · E. D. Earle

Chromosome doubling and mode of reproduction of induced tetraploids of eastern gamagrass (*Tripsacum dactyloides* L.)

Received: 12 February 1997 / Revision received: 18 February 1998 / Accepted: 13 March 1998

Abstract Eastern gamagrass, (*Tripsacum dactyloides* L.) is a perennial, warm-season grass that is being developed as a forage plant. Shoots were derived from callus initiated from immature embryos and immature inflorescences of diploid (2n=2x=36) gynomonoecious eastern gamagrass. These shoots were induced to microtiller in the presence of 3 mg/l benzyladenine. Amiprophosmethyl (10, 15, or 20 μM) was applied to 27 microtillers for 3–5 days to induce chromosome doubling. All 14 surviving plants were tetraploid, (2n=4x=72), as determined by flow cytometry or chromosome counts. These plants were morphologically normal and produced seed. Test crosses were made with a known diploid. Flow cytometry and chromosome counts showed that the progeny were triploid, proving that the induced tetraploids reproduce sexually.

Key words Eastern gamagrass · *Tripsacum* · Chromosome doubling · Tissue culture

Abbreviations *APM* Amiprophosmethyl · *BA* 6-Benzyladenine · *GSF* Gynomonoecious sex form · *MS* Murashige and Skoog · *MSF* Monoecious sex form · *NAA* Naphthaleneacetic acid

Introduction

Eastern gamagrass, (*Tripsacum dactyloides* L.) is a perennial, C4, warm-season, forage grass. Its commercial use has been delayed because of seed production problems. Eastern gamagrass usually has a monoecious sex form (MSF), in which the apical two-thirds to three-fourths of the racemes are composed of paired male spikelets with one to several solitary female spikelets in the basal portion of the racemes (Dewald et al. 1987). Gamagrass naturally occurs primarily as diploids or tetraploids. The diploids (2n=2x=36) reproduce sexually, and the tetraploids (2n=4x=72) reproduce by facultative apomixis (Farquharson 1955; Burson et al. 1990; Sherman et al. 1991).

In 1981, a high-seed-producing, diploid gynomonoecious sex form (GSF) was found which has the potential to increase seed production 20-fold; it was designated GSF-I. This form differs from the normal monoecious plants by having perfect rather than staminate paired spikelets in the terminal portion of the inflorescence, paired spikelets each with two pistillate florets and rudimentary stamens in the mid section, and two, instead of one, functional pistillate florets in the basal spikelets (Dewald and Dayton 1985).

The objective of this work was to develop a method to double the chromosome number of a diploid GSF eastern gamagrass to the tetraploid level and to determine whether such tetraploid plants reproduce sexually or apomictically. It would be useful to have tetraploid GSF lines to take advantage of apomixis, the robust nature of natural tetraploids, and the increased seed production of the GSF trait in breeding programs. Evaluation of any useful changes in the gynomonoecious seed head at the tetraploid level was also of interest. In addition, development of a chromosome-doubling technique for eastern gamagrass would be useful as a component of anther culture systems.

Communicated by J. M. Widholm

P. R. Salon (✉)
USDA NRCS Big Flats Plant Materials Center RD1,
Box 360A Rt 352,
Corning, NY 14830, USA
Fax: +1-607-5628516

E. D. Earle
Department of Plant Breeding and Biometry, Cornell University,
252 Emerson Hall, Ithaca, NY 14853-1901, USA

Materials and methods

Plant materials

The *T. dactyloides* L. var. *dactyloides* plants used are listed in Table 1. KS46 and OK20 are GSF plants derived from GSF-I germplasm and developed at the USDA-NRCS Manhattan Kansas Plant Materials Center (PMC) and the Agriculture Research Service in

Table 1 Identification, ploidy level, inflorescence type and use of plants in this study. Ploidy was verified by flow cytometry. (*Explant* starting material for tissue culture, *Parent/explant* plant used both as starting material and as a parent for other starting material, *Tester* plant used in test cross to test mode of reproduction, *Induced* plants converted to tetraploid level in this study)

Nursery no.	Accession no.	Ploidy	Inflorescence	Use
KS46	9050003	2n	GSF	Explant
OK20	WW-1703	2n	GSF	Parent/explant
GSF-I	PI 483447	2n	GSF	Parent/explant
511	9051316	2n	MSF	Parent
429	9051292	2n	MSF	Tester
OK20×(GSF-I×511)	–	2n	MSF	Explant
OK20×(GSF-I×511)-4X	9051767	4n	MSF	Induced
OK20-4X	9051769	4n	GSF	Induced
GSF-I-4X	PI 591482	4n	GSF	Induced
KS46-4X	9051774	4n	MSF	Induced

Woodward, Okla, respectively. Plants 511 and 429 were germplasm collected in Oklahoma and evaluated at the Big Flats PMC. The cross OK20×(GSF-I×511) was made at the Big Flats PMC.

Callus initiation and maintenance

Immature seeds (2–3 weeks following pollination) from the diploid cross OK20×(GSF-I×511) were surface sterilized for 20 min in 30% (vol/vol) bleach (1.6% NaOCl) plus 0.1% Tween 20 and rinsed three times with sterile distilled, deionized water. The embryos (1–2 mm in length) were aseptically removed and placed on MSDI medium.

Young unemerged inflorescences of GSF-I were taken from the early boot stage, when the inflorescences were 1–3 cm long. The outer leaves were removed, and the remaining inner tissue surface sterilized as described above. A longitudinal cut was then made aseptically through the remaining leaves, exposing the young inflorescence which was cut into 3- to 5-mm pieces and placed on MSDI medium.

MSDI medium contained MS salts (Murashige and Skoog 1962) 3.3 mg/l dicamba, 0.4 mg/l thiamine, 100 mg/l inositol, 2.3 g/l proline, and 2% sucrose. The pH was adjusted to 5.8 prior to autoclaving, and 2.2 g/l of gelrite was used as the solidifying agent. Cultures were maintained in 100×15 mm plastic Petri dishes containing approximately 25 ml of medium. They were grown in the dark at 25°C and transferred to fresh medium every 2 weeks. The callus was maintained for 3 months prior to being transferred to regeneration medium.

Shoot initiation and microtillering

Shoots were induced by a two-step procedure. Callus was placed on medium containing MS salts, 0.4 mg/l thiamine, 100 mg/l inositol, 3% sucrose, supplemented with 4 mg/l 6-benzyladenine (BA), in the dark for 2 weeks and then transferred to medium without growth regulators in the light. To induce microtillering, individual shoots were placed on the same medium supplemented with 3 mg/l BA in the light. The light was provided by a mixture of cool white and gro and sho fluorescent bulbs (General Electric) at 80 μM photons $\cdot m^{-2} \cdot s^{-1}$, in a 16-h daylength.

Chromosome doubling

Shoots initiated from embryo-derived callus of the diploid MSF (OK20×(GSF-I×511) were used to develop the chromosome-doubling technique. Twenty-seven shoots separated from actively microtillering cultures were placed in three petri dishes (9 shoots/plate) containing MS salts, 0.4 mg/l thiamine, 100 mg/l inositol, 3% sucrose, and 3 mg/l BA. Five milliliters of a 10, 15, or 20 μM solution of amiprophosnethyl (APM), a mitotic inhibitor obtained from Bayer (Leverkusen, Germany), was pipetted into each petri dish. APM was dissolved in a few drops of acetone and diluted with water. The shoots were incubated in the dark at 25°C for either 5 days (10 μM APM) or 3 days (15 and 20 μM APM). After incubation, the shoots were rinsed three times in sterile distilled, deionized water and placed on fresh medium for up to 3 weeks. After the shoots had recovered from the treatment and resumed meristematic growth, they were transferred to medium containing MS salts, 0.4 mg/l thiamine, 100 mg/l inositol and 3% sucrose supplemented with 1.0 mg/l naphthaleneacetic acid (NAA) for rooting.

Shoots from microtillers of GSF lines derived from inflorescences of GSF-I and KS46 were also treated with 20 μM APM to induce chromosome doubling. Some shoots derived from GSF-I and OK20 inflorescences were treated with colchicine (0.01–0.025%, dissolved in distilled deionized water and filter-sterilized) for 3 days, instead of APM.

Transfer of plantlets out of culture

Rooted plantlets were transplanted into pots in Cornell Mix, a soilless potting mixture (Sheldrake and Boodley 1973) and grown under the same conditions as the tissue cultures. They were covered with plastic bags for 1 week and then grown without bags for an additional week before transfer to a greenhouse. The plants were grown to maturity in a field.

Measurement of DNA content and chromosome number

The nuclear DNA content of the plants recovered was evaluated by flow cytometry of leaf samples (Arumuganathan and Earle 1991). Controls of diploid gamagrass and chicken red blood cells (2.33 pg DNA/nucleus; Galbraith et al. 1983) were run at the same settings and time to standardize results. The ploidy level was confirmed on all plants by counting the chromosome number in at least three root tip cells/plant (Kindiger 1993).

Determination of mode of reproduction

Some plants were dug from the field at the end of December and brought into a greenhouse to flower. They were given a 14-h day at

Fig. 1 Cluster of shoots (2.5 cm long) separated from a microtillering culture for treatment with APM

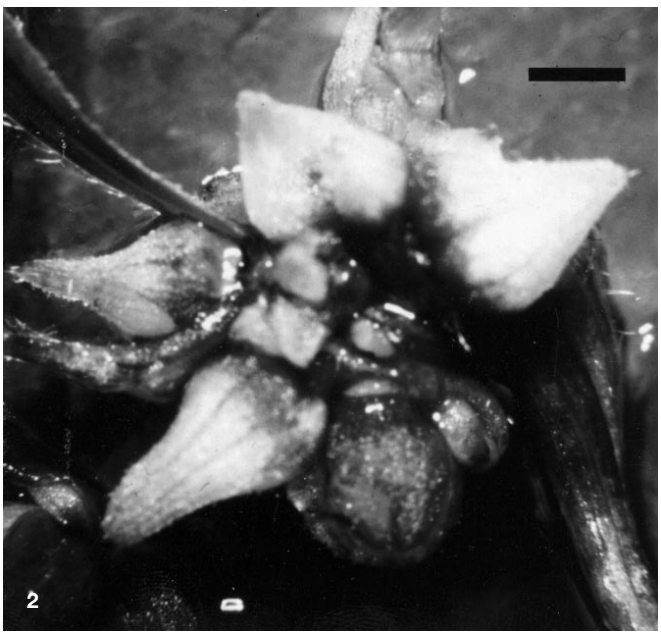
Fig. 2 Swollen meristems on tillers 5 days after APM treatment (*scale bar* 3.5 mm)

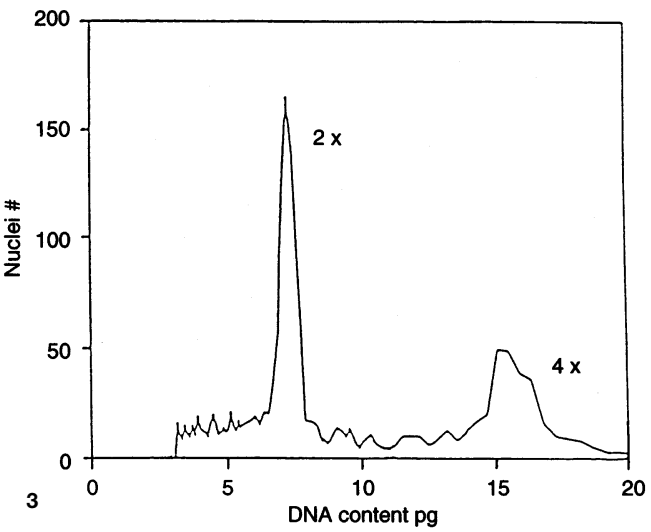
Fig. 3 Histogram showing relative DNA content of diploid starting material (7.3 pg/nucleus) and an induced tetraploid (15.1 pg/nucleus)

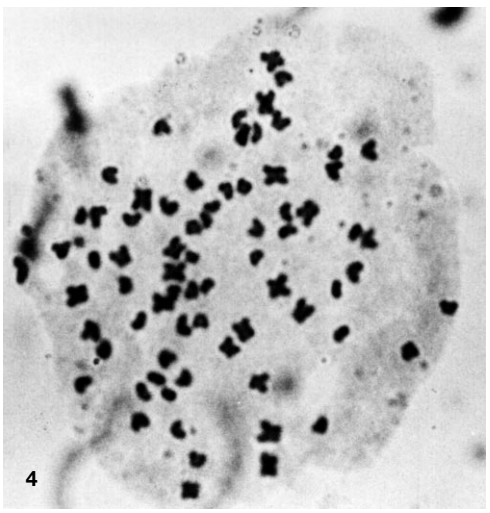
Fig. 4 A root tip cell from an induced tetraploid with 72 chromosomes

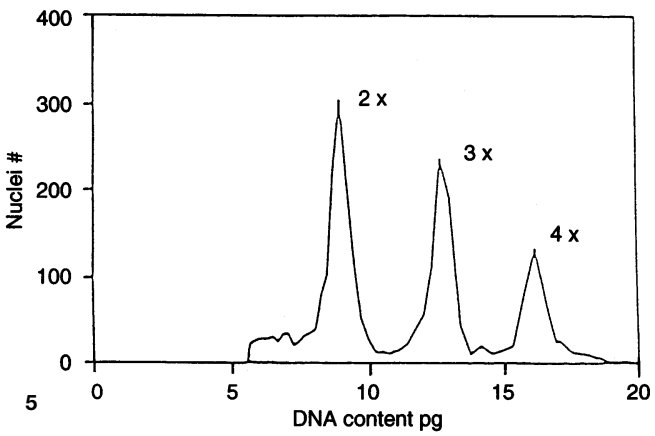
Fig. 5 A composite histogram showing relative DNA content of the diploid parent (8.5 pg/nucleus), induced tetraploid parent (15.9 pg/nucleus), and triploid progeny (12.4 pg/nucleus)

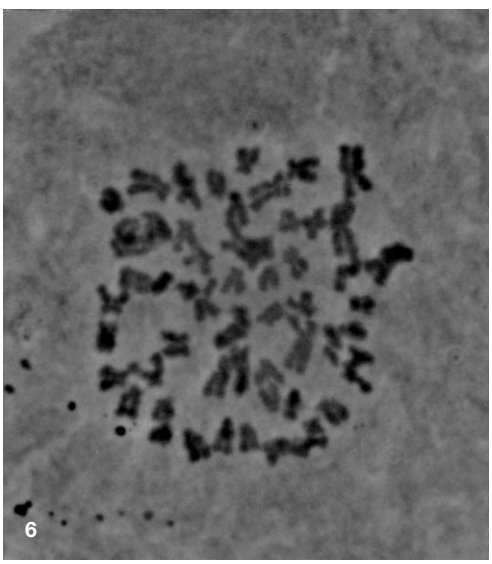
Fig. 6 A root tip cell from a triploid with 54 chromosomes, derived from a cross between the induced tetraploid and a known diploid (photograph Jie Xu)




200
150
100
50
0
Nuclei #
2 x
4 x
0
5
10
15
20
DNA content pg




400
300
200
100
0
Nuclei #
2 x
3 x
4 x
0
5
10
15
20
DNA content pg



150 μM photons · m^{-2} · s^{-1} and kept at a minimum temperature of 18°C. Flowering began 3 months later. These plants included a diploid MSF 429, and an induced tetraploid GSF-I-4X, four induced tetraploid MSF plants heterozygous for the GSF trait OK20×(GSF-I×511)-4X.

Crosses were made between induced tetraploid GSF-I-4X and diploid 429 so that progeny could be evaluated for ploidy level to determine the mode of reproduction. Sexually produced progeny would be triploid, while apomictic progeny would be tetraploid. The nuclear DNA content of 12 progeny was evaluated by flow cytometry to estimate the ploidy level. To confirm the flow cytometry findings, chromosomes from root tips of several progeny were counted.

Results and discussion

Callus suitable for subculture was induced on approximately 40% of the immature embryos and 50% of the immature inflorescence explants placed on MSDI medium. Because shoot regeneration from callus was inconsistent, a microtillering system was employed to obtain large quantities of actively growing shoots for application of APM or colchicine (Fig. 1). Individual callus-derived shoots placed on medium containing 3 mg/l BA showed extensive proliferation of microtillers, with a three- to fourfold increase in tillering in 30 days.

In vitro application of APM to microtillers induced doubling of chromosome number from diploid (2x=2n=36) to tetraploid (2n=4x=72). Twenty-seven tillers from embryo-derived callus of OK20×(GSF-I×511)-4X were treated with 10, 15, and 20 μM of APM. After 5 days, the actively dividing meristem appeared swollen (Fig. 2). After 17 days, all treatments showed some mortality and reduced vigor on some of the surviving shoots. The survival from the 10, 15, and 20 μM treatments was 66%, 30%, and 50%, respectively. All 14 surviving plants were tetraploid.

Tetraploid GSF plants were obtained from shoots derived from immature inflorescences. Two tetraploids of GSF-I and one of KS46 were identified among 43 plants tested after treatment of microtillers with a 20 μM solution of APM. Five GSF-I tetraploids were recovered from 24 plants tested after applying 0.01–0.025% colchicine solutions to microtillers. In addition, two tetraploids (one GSF-I, one OK20) were regenerated after applying 0.025% colchicine to 20 pieces of callus as described by Wan et al. (1991).

APM had several advantages over the use of colchicine. Less contamination was seen than on shoots treated with colchicine, perhaps because of the acetone in the APM treatment. Furthermore, APM is reported to be safer for handling (Wan et al. 1991), while colchicine must be handled carefully because of its harmful effects on humans.

Colchicine at the 0.05% level was phytotoxic, with no shoots surviving the treatment. A dosage of colchicine less than 0.05% can be selected which is lethal to many shoots. The efficiency of the microtillering system allows for the generation and treatment of large numbers of tillers allowing for the recovery of doubled plants from the remaining surviving tillers.

After plants from the various treatments were transferred to soil, their leaf nuclear DNA content was analyzed by flow cytometry. All tetraploid plants were retested to verify the increase in DNA content. Figure 3 shows a histogram with a diploid and an induced tetraploid in which the diploid, OK20×(GSF-I×511), has a DNA content of 7.3 pg/nucleus while the tetraploid has 15.1 pg/nucleus. G2 peaks were not apparent because the plants were not growing vigorously and the leaf samples were not taken from a meristematic region. The average nuclear DNA content of all the induced tetraploids from OK20×(GSF-I×511) was 14.8±0.4 pg/nucleus. The shift to the tetraploid DNA level was correlated with a doubling of the number of chromosomes. Figure 4 shows a tetraploid cell with 72 chromosomes from a plant recovered from the 10 μM APM treatment.

When the induced tetraploids were grown to maturity, they produced morphologically normal, fertile seed heads. To ascertain the mode of reproduction of these plants, test crosses were made between an induced tetraploid and a natural diploid. The nuclear DNA values for the test cross parents were determined. The induced tetraploid parent GSF-I-4X was found to have a DNA value of 15.9 pg/nucleus; the natural diploid 429 had 8.5 pg/nucleus. The average DNA content of the ten offspring evaluated was 12.4±0.6 pg/nucleus. This value is intermediate between the diploid and tetraploid value, indicating that the progeny are triploid, as expected from a sexual cross. Figure 5 is a composite histogram illustrating nuclear DNA content of the parents and one of the triploid offspring of this tetraploid×diploid cross. The flow cytometry results were confirmed by root tip chromosome counts; nine progeny plants were found to have a minimum of three cells with 54 chromosomes, the triploid number (Fig. 6). One plant had a variable number of chromosomes between 36 and 54.

A subsample of seed was taken from open-pollinated induced tetraploids to evaluate the percentage of spikes that yielded normal-appearing seed. An average seed set of 60.7% was observed. Although this seed set is higher than usually expected from an induced autotetraploid, a seed set of 63% has been reported for colchicine-induced cereal rye autotetraploids (Müntzing 1951). The high seed set in this case could be due to outcrossing from pollen in the surrounding nursery containing natural diploids and tetraploids. The protogynous flowering of eastern gamagrass will also reduce the chances of self-pollination from the same inflorescence. An exact measure of the fertility of the induced tetraploids was not obtained; however, progeny were readily recovered from controlled crosses between induced tetraploids (86 plants) and from crosses between induced tetraploids and natural tetraploids (305 plants). The different number of progeny from the two types of crosses is not meaningful, since more crosses were made between induced and natural tetraploids. Fifty-three progeny from these crosses were sampled; all were tetraploid as measured by flow cytometry. It appears that these induced tetraploids have a fertility level sufficient to be utilized in a breeding program.

Chromosome doubling of embryogenic callus of diploid *Tripsacum* was accomplished by Leblanc et al. (1995) using 0.05–0.2% colchicine. While various *T. dactyloides* subspecies were used, none were *T. dactyloides* var. *dactyloides*, the subspecies native to North America. All were monoecious, unlike the diploid GSF germplasm used in this study. Leblanc et al. (1995) also found that their induced tetraploids reproduced sexually.

The GSF trait increases the seed production potential of eastern gamagrass 20-fold and is being utilized in several breeding programs. The trait is governed by a recessive gene, and the GSF inflorescence produces little pollen, resulting in inadequate seed set when GSF plants are naturally intercrossed. This results in a lack of true-breeding GSF lines in seed production fields.

The transfer of the GSF trait from the diploid level to the tetraploid level therefore has several advantages. Crosses of the induced tetraploid sexual GSF plants with apomictic MSF tetraploid plants used as pollen parents will produce fertile progeny that could be used in breeding systems to fix the GSF trait with apomixis. An apomictic GSF plant will breed true regardless of the gamagrass pollen source. Eastern gamagrass shows a high degree of shattering, so breeding apomixis into the genotypes used in seed production fields would reduce genetic drift and the production of off types from germinating shattered seed. This would increase the length of time seed production fields can be maintained.

On the other hand, sexually reproducing tetraploid eastern gamagrass will permit recombination at the tetraploid level. The resulting unique and heterotic genotypes may also be fixed with apomixis. The tetraploid gamagrass plants found along the northeast and mid Atlantic coastal areas are larger, more robust and flower around 3 weeks later than the diploids obtained from the midwest. These tetraploid plants appear to decline in vigor more quickly than the diploids, with center dieback. It would be beneficial to be able to improve longevity in these tetraploids by conventional breeding methods. Sexual tetraploids will also be useful for future genetic studies of the inheritance of apomixis and for the study of the inheritance of the GSF trait at the tetraploid level.

The induced sexual GSF tetraploid (PI 591482) was released as germplasm by the USDA-NRCS and Cornell University in January 1996 (Salon and Pardee 1996).

References

Arumuganathan K, Earle ED (1991) Estimation of nuclear DNA contents of plants by flow cytometry. Plant Mol Biol Rep 9:221–231

Burson BL, Voigt PW, Sherman RA, Dewald CL (1990) Apomixis and sexuality in eastern gamagrass. Crop Sci 30:86–89

Dewald CL, Dayton RS (1985) Registration of gynomonoecious germplasm (GSF-I and GSF-II) of eastern gamagrass. Crop Sci 25:715

Dewald CL, Burson BL, deWet JMJ, Harlan JR (1987) Morphology,inheritance, and evolutionary significance of sex reversal in *Tripsacum dactyloides* (Poaceae). Am J Bot 74:1055–1059

Farquharson LI (1955) Apomixis and polyembryony in *Tripsacum dactyloides*. Am J Bot 42:737–743

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049–1051

Kindiger B (1993) A technique for the preparation of somatic chromosomes of maize. In: Freeling M, Walbot V (eds) The maize handbook. Springer, Berlin Heidelberg New York, pp 481–483

Leblanc O, Duenas M, Hernandez M, Bello S, Garcis V, Berthaud J, Savidan Y (1995) Chromosome doubling in *Tripsacum*: the production of artificial, sexual tetraploid plants. Plant Breed 114:226–230

Müntzing A (1951) Cyto-genetic properties and practical value of tetraploid rye. Hereditas 37:17–84

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473–497

Salon PR, Pardee WD (1996) Registration of SG4X-1 germplasm of eastern gamagrass. Crop Sci 36:1425

Sheldrake R Jr, Boodley JW (1973) Cornell peat-lite mixes for commercial plant growing. Coop Ext IB 43. NY State College Agric Life Sci, Cornell Univ, Ithaca, NY

Sherman RA, Voigt PW, Burson BL, Dewald CL (1991) Apomixis in diploid×triploid *Tripsacum dactyloides* hybrids. Genome 34: 528–532

Wan Y, Duncan DR, Rayburn AL, Petolino JF, Widholm JM (1991) The use of antimicrotubule herbicides for the production of doubled haploid plants from anther-derived maize callus. Theor Appl Genet 81:205–211